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ABSTRACT

The presence of autoantibodies to multiple islet autoantigens confers high risk for development of Type 1 diabetes. Four major autoantigens are established (insulin, glutamate decarboxylase, IA-2, and zinc transporter-8), but the molecular identity of a fifth, a 38kDa membrane glycoprotein (Glima), is unknown. Glima antibodies have been detectable only by immunoprecipitation from extracts of radiolabeled islet or neuronal cells. We sought to identify Glima to enable efficient assay of these autoantibodies. Mouse brain and lung were shown to express Glima. Membrane glycoproteins from extracts of these organs were enriched by detergent phase separation, lectin affinity chromatography and SDS-PAGE. Proteins were also immunoaffinity purified from brain extracts using autoantibodies from diabetic patients' sera before SDS-PAGE. Eluates from gel regions equivalent to 38kDa were analyzed by LC-MS/MS for protein identification. Three proteins were detected in samples from the brain and lung extracts, and in the immunoaffinity purified sample, but not the negative control. Only tetraspanin-7, a multipass transmembrane glycoprotein with neuroendocrine expression, had physical characteristics expected of Glima. Tetraspanin-7 was confirmed as an autoantigen by demonstrating binding to autoantibodies in Type 1 diabetes. We identify tetraspanin-7 as a target of autoimmunity in diabetes, allowing its exploitation for diabetes prediction and immunotherapy.

INTRODUCTION

Detection of circulating autoantibodies to pancreatic islets (1), and identification of their molecular targets (2), has allowed development of high throughput autoantibody assays for clinical diagnosis of Type 1 diabetes and identification of individuals at risk for disease. Evidence from both animal studies and human trials indicate that Type 1 diabetes may be prevented in individuals at risk (3; 4). Hence, a range of therapies to interfere with immune responses has proved effective in preventing disease development in animal models of diabetes (5) and in slowing the loss of beta cell function occurring in the months following disease diagnosis in man (6-8). There is now a focus on development of procedures to interfere specifically in immune responses that cause Type 1 diabetes, requiring knowledge of the major targets of the autoimmune response. There is no single common autoimmune target and individuals differ in antigen specificity of autoimmune responses they develop. Four major humoral autoantigens have been identified in Type 1 diabetes by defining the specificity of autoantibodies in the disease: insulin (9), glutamate decarboxylase (10), IA-2 (11), and zinc transporter-8 (ZnT8) (12). Autoantibodies to a fifth, a 38kDa glycosylated membrane protein (Glima), have been detected in 19-38% of Type 1 diabetic patients, with significantly higher prevalence (up to 50%) in children (13-15). The molecular identity of Glima has for many years proved elusive, hampering the characterization of autoimmunity to the protein and the development of sensitive, specific autoantibody assays. Glima is expressed in pancreatic beta and neuronal cell lines, is hydrophobic, heavily N-glycosylated, having affinity for the lectin wheat germ agglutinin, and has a core protein backbone of approximately 22kDa (13-15). The aim of

this study was to take advantage of these known physical properties to prepare Glima-enriched extracts for identification of the autoantigen by mass spectrometry.

RESEARCH DESIGN AND METHODS

Patients. Serum samples were obtained from 40 Type 1 diabetic patients (12- 6 y) within 6 months of diagnosis from clinics in West Yorkshire with informed consent for screening for high-titer Glima antibodies, 94 additional patients (12-63 y) for assay verification and 52 non-diabetic individuals as negative controls. Approval for analysis of autoantibodies in sera from these individuals was obtained from the Yorkshire and the Humber – Bradford Leeds Research Ethics Committee.

Screen of type 1 diabetic patient sera for Glima antibodies. Glima antibodies were detected by a modification of immunoprecipitation assays previously described (13-15) using the neuronal mouse cell line GT1.7 as source of antigen. Endogenous proteins in GT1.7 cells were labeled by incubation in methionine-free DMEM medium containing 4 MBq/ml ^{35}S -methionine for 7 h at 37°C. Cells were washed with Hepes buffer (10 mM Hepes, pH 7.4, 150 mM NaCl, 10 mM benzamidine) and stored at -80°C. Frozen cell pellets were extracted in Hepes buffer containing 2% Triton X-100 for 2 h on ice and insoluble material removed by centrifugation at 15,000 g for 15 min at 4°C. Membrane glycoproteins were isolated by incubating cell extracts with wheat germ agglutinin-agarose on ice for 30 min and, after washing in Hepes buffer containing 0.5 mM methionine, 100 mg/l bovine serum albumin and 0.5% Triton X-100, eluted in the same buffer containing 0.5 M N-acetyl glucosamine. Aliquots (20 μl) of eluate containing 3 \times

10^5 cpm of radiolabeled protein were incubated with 5 μ l of test sera for 18 hours at 4°C and immune complexes captured on 5 μ l of protein A-Sepharose. Immunoprecipitated proteins were eluted in 15 μ l of SDS-PAGE Loading Buffer (Novex, Life Technologies, Paisley, UK) with heating at 90°C for 5 min and subjected to SDS-PAGE on 12% polyacrylamide gels. After electrophoresis, gels were incubated in 40% v/v methanol, 2.5% v/v acetic acid and subsequently in Enlightning autoradiographic enhancer (Perkin-Elmer, Coventry, UK), each for 30 min. Gels were dried and contacted with X-ray film (BioMax MR film; Kodak, Watford, UK) for up to two weeks. After exposure, X-ray film was developed to detect radiolabeled proteins specifically immunoprecipitated by sera from Type 1 diabetic patients, with bands detected in the 38,000 relative molecular mass (M_r) region indicating positivity for Glima antibodies.

Tissue expression screen. To identify mouse organs containing highest levels of Glima for use in antigen purification, competitive binding studies were performed using detergent extracts of organs as unlabeled competitors with 35 S-methionine-labeled Glima for binding to Glima antibodies in serum from a high-titer Glima antibody positive patient. Mouse kidney, brain, heart, liver, thyroid, muscle, salivary gland, thymus, pancreas, spleen, adrenal, pituitary, and lung were dissected, frozen in liquid nitrogen and stored at -80°C before extraction. Tissues were homogenized in homogenization buffer (10 mM Hepes, pH 7.4, 0.25 M sucrose, 10 mM benzamidine), and membrane fractions sedimented by centrifugation at 15,000 g for 30 min at 4°C. Supernatants were removed and pellets extracted in 2% Triton X-100 extraction buffer for 2 h on ice. Extracts were centrifuged at 15,000 g for 30 min at 4°C and supernatants collected. The protein

concentrations of extracts were determined using the Pierce BCA protein assay kit (Thermo Fisher Scientific, Loughborough, UK).

For the competition assay, wheat germ agglutinin agarose eluates from extracts of ^{35}S -methionine-labeled GT1.7 cells were prepared as described above. Aliquots (20 μl) of GT1.7 cell glycoproteins containing 3×10^5 cpm of radiolabeled proteins were incubated for 18 h at 4°C with 5 μl of serum from patient 029 alone, or with 10 μl of detergent extracts containing 100 μg of extracted protein from each mouse tissue. Immune complexes were captured on protein A-Sepharose and processed for SDS-PAGE and autoradiography.

Partial purification of Glima from mouse brain and lung. Mouse brain and lung, shown to express Glima in the tissue screen (see Results), were homogenized in ice-cold homogenization buffer in a Dounce homogenizer and cell debris removed by centrifugation at 500 g for 5 min at 4°C . A membrane fraction was prepared by centrifugation of the supernatant at 10,000 g for 15 min at 4°C , and the pellet washed and extracted in 2% Triton X-114 extraction buffer for 2 h at 4°C . Insoluble material was removed by centrifugation at 10,000 g for 15 min at 4°C and a detergent phase prepared by heat-induced phase-separation as previously described (13). Fractions were added to wheat germ agglutinin-agarose at a ratio of 100 μl lectin-agarose to 5 mg total protein and incubated overnight at 4°C with gentle mixing. The beads were washed twice with Hepes buffer containing 0.5% Triton X-100 and twice in NOG buffer (1% n-octyl-

glucopyroside in Hepes buffer). Wheat germ agglutinin-binding proteins were eluted in 0.5 M n-acetyl-glucosamine in NOG buffer.

Eluates were concentrated using the SDS-PAGE Sample Preparation kit (Pierce, Life Technologies, Paisley, UK), solubilized in SDS-PAGE Loading Buffer (Novex, Life Technologies) for 10 minutes at 60°C and electrophoresed on 12% Bis-Tris gels in MOPS running buffer. Gels were stained with Brilliant Blue G-Colloidal Coomassie (Sigma-Aldrich, Poole, UK) and gel slices corresponding to the 38,000 M_r region excised for mass spectrometry.

Immunoaffinity purification with Glima antibodies. For immunoaffinity purification, 250 µl of pooled sera from three patients with high Glima antibodies were used with 250 µl sera from three antibody-negative individuals as negative control. Sera were incubated with Protein A-Sepharose (250 µl) for 1 h at room temperature with rolling and washed three times in borate buffer (100 mM boric acid, pH 8.3). Antibodies were cross-linked to Protein A-Sepharose with 20 mM dimethylpimelidate in borate buffer for 1 h. Unreacted sites were blocked with 20 mM ethanolamine for 10 min and washed before use.

Triton X-114 detergent phase-purified amphiphilic proteins from mouse brains prepared as above were added to the Glima antibody-positive and -negative-beads and incubated overnight at 4°C with mixing. Beads were washed with 0.5% Triton X-100 in Hepes buffer prior to elution in 2% SDS at 90°C for 10 min. The eluate was concentrated to 20 µl using the SDS-PAGE Sample Preparation Kit, subjected to SDS-PAGE and Colloidal

Coomassie gel staining as above, and gel slices in the 38,000 M_r region excised for mass spectrometry.

In-gel trypsin digestion and mass spectrometry of 38,000 M_r proteins. Gel slices representing 38,000 M_r regions of all samples were processed using the In-Gel Tryptic Digestion kit (Pierce) according to the manufacturer's instructions and the trypsin-treated extracts vacuum-dried and stored at -20°C prior to mass spectrometry. Samples were reconstituted in 30 μl of 50 mM ammonium bicarbonate for 30 min at room temperature and centrifuged at 15,000 g for 15 min to remove insoluble material. Samples were transferred to autosampler tubes and 10 μl of each analyzed by LC-MS/MS. Peptides were resolved by reversed phase chromatography on a 75 μm C18 EASY column using a linear gradient of acetonitrile in 0.1% formic acid at a flow rate of 300 nL/min over 50 min on an EASY Nano LC system (Thermo Fisher Scientific). The eluate was ionized by electrospray ionization using an Orbitrap Velos Pro machine (Thermo Fisher Scientific) operating under Xcalibur (version 2.2; Thermo Fisher Scientific) and precursor ions selected based on their intensity for sequencing by collision-induced fragmentation. The MS/MS analyses were conducted using collision energy profiles that were chosen based on the mass-to-charge ratio (m/z) and the charge state of the peptide.

Tandem mass spectra were processed into peak lists using Proteome Discoverer (version 1.3; Thermo Fisher Scientific). All MS/MS samples were analyzed using Mascot (version 2.2.06; Matrix Science, London, UK) searching the Uniprot Mus musculus database, assuming digestion with trypsin. Mascot was searched with a fragment ion mass

tolerance of 0.80 Da and a parent ion tolerance of 10.0 PPM, with oxidation of methionine and carbamidomethylation of cysteine as variable modifications. Each dataset was analyzed with a reverse FASTA database acting as a decoy.

Scaffold (version 4.3.2; Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications, which were assigned by Peptide Prophet algorithms (16; 17) and accepted at > 95.0% probability. The UniProt database was manually searched for physical characteristics of the proteins identified, including molecular weight, tissue distribution, and glycosylation. Of those identified, only one, tetraspanin-7 (Tspan7), matched the known properties of Glima (see Results) and was characterized further.

Immunohistochemistry. Tspan7 localization in rodent tissues was performed by immunohistochemistry. Sections of formalin-fixed paraffin-embedded rat brain, pituitary, pancreas, adrenal gland, lung, muscle, heart, liver, kidney, spleen, and thymus were de-waxed and subjected to epitope retrieval in a microwave pressure cooker in 10 mM citric acid pH 6.0, 0.05% Tween 20. Endogenous peroxidase activity was inhibited with 0.3% H₂O₂ and non-specific binding blocked with 25% non-immune swine serum in PBS. Primary antibody to Tspan7 (Anti-TM4SF2, Sigma-Aldrich; HPA003140) was applied at 1:1,000 dilution and incubated overnight at 4°C. Antibody labeling was detected with the Envision kit (Dako, Ely, UK) according to the manufacturer's instructions, sections counterstained in Meyers Haematoxylin (Sigma-Aldrich) and visualized by microscopy.

Cloning and expression of recombinant Tspan7. cDNA for the coding region of mouse Tspan7 was amplified by RT-PCR from the mouse islet cell line Min6 using primers (5'- GAATTCATGGCATCGAGGAGAATGG-3' and 5'- AGATCTCACCATCTCATACTGATTGGC-3') that introduce *Eco*R1 and *Bgl*II sites at the 5' and 3' ends, respectively, with the native stop codon removed to allow expression as a fusion protein with a C-terminal purification tag. The PCR product was cloned into the pFLAG-CTS expression vector for protein expression in *E. coli* BL21 cells after induction with IPTG. Expressed protein was extracted from cells with Hen Egg Lysozyme (1 mg/ml) in PBS containing 10 mM benzamidine, 1 mM PMSF for 30 min at room temperature, followed by incubation in Triton X-100 (0.1%) for 5 min and DNase (1 µg/ml) for 10 min. The lysate was centrifuged at $10,000 \times g$ for 10 min at 4°C, and the supernatant used in immunoprecipitation assays.

Tspan7 binding to autoantibodies in Type 1 diabetes. Individual Glima antibody-positive and -negative human sera (15 µl) were incubated with protein A-Sepharose (15 µl), and the immunoglobulin captured was cross-linked to beads with dimethyl pimelimidate (18). Bead-bound antibodies were incubated overnight at 4°C with Triton X-100 extracts of mouse brain, or with lysates of *E. coli* expressing recombinant mouse Tspan7. Beads were washed three times in 0.5% Triton X-100 in Hepes buffer and captured proteins subjected to SDS-PAGE and Western blotting using rabbit anti-Tspan7 antibody (anti-TM4SF; Sigma-Aldrich HPA003140) at 1:250 dilution overnight at 4°C. Immunoprecipitated Tspan7 was detected with goat anti-rabbit IgG-peroxidase (product

A0545; Sigma-Aldrich;) and SuperSignal West Pico Chemiluminescent substrate (Thermo Fisher Scientific).

Luminescent immunoprecipitation assay for detection of Tspan7 antibodies

The coding region of human Tspan7 was cloned into the pCMVTnT vector as a fusion with Nanoluciferase (Promega, Southampton, UK) at the 3' end. The construct was transfected into HEK 293 cells with ExpiFectamine (Thermo Fisher Scientific) for transient expression of antigen. Transfected cells were extracted in either 2% Triton-X114 or passive lysis buffer (Promega) and insoluble material removed by centrifugation. Triton-X-114 extracts were subjected to phase separation as described above and analysed for fusion protein by Western blotting with rabbit antibodies to Nanoluciferase (gift of Promega UK), or Tspan7 (anti-TM4SF). Luciferase expression in the cell extracts was quantified by luminometry using Nano-Glo assay reagent (Promega) and aliquots of passive lysis buffer extracts containing 10^6 light units of antigen were incubated with 5 μ l of serum at 4°C for 16 h prior to capture of antibody complexes on protein A Sepharose. Mouse brain extracts (150 μ g protein) or lysates of E coli expressing Tspan7 (250 μ g protein) were added to reactions as sources of Tspan7 to compete for antibody binding. Complexes were washed and luciferase activity immunoprecipitated determined by luminometry with Nano-Glo assay reagent.

RESULTS

Selection of Glima antibody-positive sera. To identify patients with high levels of Glima antibodies for immunoaffinity purification, sera from 40 recent-onset Type 1 diabetic patients were screened by immunoprecipitation using radiolabelled mouse GT1.7 cell extracts (Fig 1). Intense diffuse 38,000 M_r bands typical of Glima were detected for three patients, indicative of high levels of Glima antibodies (029, 037 and 110 in Fig. 1). These three sera were used for subsequent Glima characterization and purification. Weaker 38,000 M_r bands indicative of Glima antibody positivity were detected in an additional 11 patients (Fig 1).

Tissue specificity of Glima expression. To identify large organs in which Glima is expressed at suitably high levels for antigen purification, competitive binding studies were performed in which detergent extracts of normal mouse tissues acted as unlabeled competitors with radiolabeled Glima from GT1.7 cell lysates for binding to antibodies in serum from the strong Glima antibody-positive patient, 029. Extracts of brain, pituitary, and lung reduced the intensity of radiolabeled 38,000 M_r protein, indicative of Glima immunoreactivity in these tissues (Fig. 2).

Identification of Glima candidate proteins by mass spectrometry. Extracts enriched for glycosylated membrane proteins from both brain and lung were prepared using Triton X-114 phase separation of amphiphilic membrane proteins followed by wheat germ agglutinin affinity purification. Proteins migrating at 38,000 M_r by SDS-PAGE were

trypsinized and analyzed by LC-MS/MS. A total of 65 candidates in brain and 25 in lung were identified, of which 20 were common to both samples, (Supplementary Table 1). Glycosylated membrane proteins immunoprecipitated from brain extracts by antibodies in the high Glma antibody titer patients' serum pool were also subjected to SDS-PAGE and LC-MS/MS analysis and three of the 20 protein candidates common to brain and lung were present in the Glma antibody-positive sample, but not the negative control (Supplementary Table 1). These were: 1) cytoplasmic actin-1 (Actb), a ubiquitous non-glycosylated cytoskeletal protein with a predicted molecular weight of 42 kDa; 2) guanine nucleotide-binding protein G(i) subunit alpha-2 (Gnai2), a non-glycosylated membrane associated 40 kDa protein with a wide tissue distribution; and 3) tetraspanin-7 (Tspan7), a hydrophobic four-transmembrane domain protein with core molecular weight 27.5 kDa, five putative N-glycosylation sites and a neuroendocrine distribution. Tspan7 closely matched the known properties of Glma and additional studies were performed to compare properties and validate Tspan7 as the autoantigen.

Localization of Tspan7 in rat tissues. The tissue distribution of Tspan7 was determined by immunohistochemistry for comparison with patterns of Glma expression in competition experiments above. Strong immunolabeling for Tspan7 was detected in the rat brain, in particular the cerebral cortex, hippocampus, cerebellum, striatum and thalamus (Fig. 3A), in the pancreatic islets (Fig. 3B), in the anterior pituitary (Fig. 3C), and in epithelial cells lining the alveoli in the lung (Fig. 3D). These observations agree with the Glma immunoreactivity described above, and previously (13, 15). Weak Tspan7 immunolabelling was also found in cells of the adrenal gland (Fig. 3E). No evidence of

Tspan7 expression was found in the exocrine pancreas (Fig. 3B), muscle, heart, liver, kidney, spleen, or thymus (not shown).

Immunoprecipitation of Tspan7 by antibodies in Type 1 diabetes. To demonstrate that Tspan7 is a target for autoantibodies in Type 1 diabetes, extracts of mouse brain and lysates of *E. coli* expressing recombinant mouse Tspan7 were subject to immunoprecipitation with Glma antibody-positive and -negative sera followed by Western blotting with a rabbit antibody to Tspan7. A 38,000 M_r band representing Tspan7 was selectively immunoprecipitated from mouse brain detergent extract by three Glma antibody-positive sera, but not by control samples. The bands detected co-migrated with the brain lysate control (Fig. 4A). Antibodies in Type 1 diabetic patients' sera also specifically immunoprecipitated Tspan7 from bacterial lysates containing recombinant protein (Fig 4B). Here, the protein migrated at approximately 22,000 M_r, consistent with a lack of glycosylation in bacteria (Fig. 4B). The results confirm Tspan7 as a target of autoantibodies in Type 1 diabetes.

Analysis of Tspan7 antibodies by luminescence immunoprecipitation assay

Patients screened for Glma antibodies were analyzed for Tspan antibodies by immunoprecipitation of recombinant Nanoluciferase-tagged human Tspan7. Western blotting with rabbit polyclonal antibodies to both Nanoluciferase and Tspan7 detected diffuse 38,000 M_r bands (the expected size of the non-glycosylated fusion protein) as the dominant immunoreactivity in cells transfected with the construct, with additional bands at approximately 80,000 M_r (Fig 4C). The 38,000 M_r protein partitioned into the

detergent on temperature-induced phase separation in Triton X-114. Transfected cell extracts were used in immunoprecipitation studies with normal control sera or with sera from Glima antibody-positive and -negative Type 1 diabetic patients. All but one (V015) of the controls (n=52) had low Tspan7 antibodies (Fig 4D). Four patients with high Glima antibodies (Fig 1) also immunoprecipitated high luciferase activity in the Tspan7 antibody assay (Fig 4D) and significantly higher levels of Tspan7 antibodies were found in Glima antibody-positive patients than those Glima antibody negative ($p < 0.0001$; Mann Whitney U test). In competition assays, natural or recombinant Tspan7 in brain or E coli extracts partially (control sample V015), or completely (Glima antibody positive Type 1 diabetic patients) blocked antibody binding to the Nanoluciferase-Tspan7 construct (Fig 4E). Control V015 did not bind Tspan7 from mouse brain extracts when tested in the Western blotting assay. A second set of 94 recent onset Type 1 diabetic patients were also tested in the Tspan7 antibody assay. Using a cut-off of mean + 3SD of controls (omitting the outlier), 40 (43%) were positive for Tspan7 antibodies (Fig 4D).

DISCUSSION

Autoantibodies to “Glima” in Type 1 diabetes were first reported in 1996 (13), but its molecular identity has since then remained unknown. We used mass spectrometry of Glima-enriched fractions of brain and lung to search for likely candidates for Glima. LC-MS/MS analysis identified 65 proteins in 38,000 M_r gel samples of amphiphilic membrane glycoproteins from brain and 25 from lung, of which 20 were common to both (Supplementary Table 1). Additional LC-MS/MS analysis of immunoaffinity-purified proteins isolated from brain extracts using protein A Sepharose-coupled immunoglobulins from Glima antibody-positive sera (with similar preparations from Glima antibody-negative sera as a negative control) further narrowed down potential candidates for testing of autoantigenicity. Only six proteins detected in the brain or lung extracts were also present in the immunoaffinity purified sample but absent in the negative control. Of these, five (Actb, Gnai2, Sfxn5, Kctd12 and Tuba1b) had considerably higher predicted molecular weight (>36kDa) than expected for the non-glycosylated Glima protein (approximately 22kDa; reference 15). Furthermore, Actb, Gnai2 and Tub1a are ubiquitous cytoplasmic proteins lacking the amphiphilic characteristics expected of Glima. Sfxn5 and Kctd12 were not detected in the lung extract. Tspan7 was consequently the most promising candidate for Glima identified in the LS-MS/MS analyses.

Tspan7 is a member of the tetraspanin family, members of which share structural characteristics of four transmembrane domains, with one short (EC1) and one long (EC2)

extracellular loop (19). Four of the putative N-glycosylation sites are contained within the EC2 domain with a further site located in EC1. The presence of multiple transmembrane domains and N-glycosylation sites within Tspan7, is consistent with the hydrophobic properties and heavy N-glycosylation previously reported for Glma (13,15). There are four amino acid differences between mouse and human Tspan7, all being located in the long extracellular loop. The tissue distribution of Tspan7 expression has not been widely investigated but analysis of transcriptional activity of the Tspan7 gene has shown restricted tissue distribution with high levels being detected in regions of the adult mouse brain and lung (20). In the pancreas, Tspan7 is found specifically in the islets of Langerhans (21). Functionally, tetraspanin family members are involved in mediating signal transduction events and have been noted to regulate cell development, activation, growth, and motility through the trafficking of other transmembrane proteins (22). Both the extracellular and intracellular domains are able to interact with other proteins, and a number of tetraspanins bind integrins, thereby forming links with the actin cytoskeleton (23). Mutations in the Tpan7 gene are associated with X-linked mental retardation and neuropsychiatric diseases, potentially as a result of impaired ability of the actin cytoskeleton to drive neurite outgrowth (24). The ability of tetraspanins to form complexes with other membrane and cytosolic proteins may explain co-purification of multiple protein fragments identified in the LC-MS/MS analysis.

Proteins immunoprecipitated by Glma antibody-positive sera from brain extracts, or from bacterial extracts containing recombinant Tspan7, also bound rabbit antibodies to Tspan7 by Western blotting, confirming Tspan7 as the target of the antibodies. Glma

autoantibodies bound both the glycosylated natural 38,000 M_r Tspan7 in brain and the 22,000 M_r non-glycosylated form of the protein expressed in *E. coli*. A luminescence-based immunoprecipitation system (LIPS; 25) using Nanoluciferase-tagged human Tspan7 expressed in mammalian cells showed that patients with high levels of Glima autoantibodies were also strongly positive in the anti-Tspan7 LIPS. The relationship between Glima- and Tspan-autoreactivity is imperfect, which may in part be the consequence of difficulties in ascertaining whether or not diffuse Glima bands are present on autoradiographs of immunoprecipitation reactions (see Fig 1). Non-diabetic individuals had low levels of Tspan7 antibodies, with the exception of one strongly positive control. Antibodies in this control serum bound poorly to natural Tspan7 from mouse brain extracts suggesting that antibodies in this particular sample may bind epitopes not displayed on the natural protein. The SDS-PAGE gel migration of the fusion protein indicated that the majority of recombinant luciferase-tagged protein was not subject to heavy glycosylation found on the natural protein, indicative of incorrect membrane insertion, protein folding or intracellular targeting of the fusion protein required for appropriate post-translational modification. Incorrect folding or lack of glycosylation may reveal antibody epitopes not normally displayed on the natural Tspan7. Further optimization of Tspan7 expression should permit the development of high throughput assays for the detection of diabetes-associated Tspan7 autoantibodies with high sensitivity and specificity.

Autoimmunity to major autoantigens in Type 1 diabetes appears within the first five years of life in at-risk children (26), with individual immune responses developing

sequentially rather than simultaneously (27). Autoimmunity in the disease is therefore progressive, with the order of appearance of autoimmune responses to individual antigens differing between individuals, and diversification of the immune response being essential for disease progression; individuals who develop autoimmunity to only single autoantigens rarely develop disease (28). The optimum strategy currently adopted for assessing disease risk is to screen individuals for the presence of autoantibodies to multiple islet autoantigens. The inclusion of Tspan7 antibodies in the screen may improve sensitivity and specificity of disease prediction in large populations, and will provide a fuller description of the major autoimmune responses that are developing in that individual, necessary for guiding the selection of autoantigen-specific immunotherapeutics to prevent the disease.

AUTHOR CONTRIBUTIONS

KAM designed the study, researched and analyzed data and wrote the manuscript; CCR, AR, CB, DL, VL, LP, DM and RF researched and analyzed data. MRC designed the study, researched and analyzed data and contributed to the writing of the manuscript. All authors reviewed and edited the manuscript and approved the final version for submission. MRC is guarantor of the study and, as such, had full access to all data and takes responsibility for the integrity and accuracy of the data analysis.

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Figure legends

Figure 1. Autoradiogram showing a screen of serum samples from Type 1 diabetic patients for Glima antibodies by immunoprecipitation of the 38,000 M_r protein from extracts of GT1.7 cells with detection by SDS-PAGE and autoradiography. A normal control serum (-ve) and a Type 1 diabetic patient previously determined to be positive for Glima antibodies (SL) were included in the assay. The location of Glima on the autoradiograph is marked. An indication of whether the samples were determined to be negative (-) or positive (+) for Glima antibodies is shown under each lane of the figure.

Figure 2. Autoradiograph of tissue expression screen demonstrating competition for Glima antibody binding to Glima antibody positive serum “029” by proteins in Triton X-100 detergent (100 µg) extracts of normal mouse tissues. Serum from a non-diabetic individual was included as a negative control. Reduced intensity of the 38,000 M_r band is indicative of Glima immunoreactivity in that tissue.

Figure 3. Immunohistochemical analysis of Tspan7 expression in rat tissues. Sections of formalin fixed, paraffin-embedded rat tissues were labeled with rabbit antiserum to Tspan7 and labeling detected by a peroxidase/DAB-based system with positive labeling detected as brown staining under the microscope. Representative images of labeling of tissue sections of brain (A), pancreas (B), pituitary (C), lung (D), and adrenal (E) are shown.

Figure 4. A. Tspan7 labeling of Western blots of mouse brain proteins immunoprecipitated by antibodies in sera from Glima antibody-negative and positive

recent onset Type 1 diabetic patients. The migration of molecular weight markers ($10^{-3} \times M_r$) on the gel and the localization of Tspan7-specific bands at 38,000 M_r are marked. **B.** Tspan7 labeling of Western blots of proteins from lysates of Tspan7-expressing *E.coli* immunoprecipitated by antibodies in sera from Glima antibody-negative and -positive recent onset Type 1 diabetic patients. The localization of Tspan7-specific bands at approximately 22,000 M_r is marked; migration of molecular weight markers as in A. In both panel A and B, IgG heavy chains (50,000 M_r), light chains (25,000 M_r) and cross-linked immunoglobulin (>100,000 M_r) from all serum samples were also detected on the blot as a consequence of cross-reactivity with the peroxidase-conjugated anti-rabbit detection antibody. **C.** Tspan7 was expressed as a fusion protein with Nanoluciferase and Triton X114 extracts of cells were subject to heat-induced phase separation. Detergent and aqueous phases were subject to SDS-PAGE and Western blotting with antibodies to Nanoluciferase (NanoLuc) or Tspan7. The migration of molecular weight markers are shown ($10^{-3} \times M_r$). **D.** Detergent extracts of Nanoluciferase were immunoprecipitated with normal control sera (n=30), Glima antibody (Ab) positive Type 1 diabetic (T1D) patients' sera (n=15) and Glima antibody-negative Type 1 diabetic patients' sera and luciferase activity associated with each immunoprecipitate determined by luminometry. Data are plotted as luciferase activity immunoprecipitated in kilo light units (kLU) and sample codes for control or diabetic individuals with high levels of antibodies are shown. **E.** Samples from control individuals or Glima antibody-positive Type 1 diabetic patients were tested for competitive binding by natural or recombinant Tspan7 in the LIPS by performing the immunoprecipitations in the absence (black bars) or presence of 150 μ g of mouse brain extract (white bars) or 250 μ g of lysates of *E coli* expressing recombinant

Tspan7 (hatched bars). Assays were performed in triplicate. Addition of brain and E coli lysate significantly blocked antibody binding for all samples ($p < 0.0001$; ANOVA with Dunnett's correction for multiple comparisons), with the exception of control sample CH.

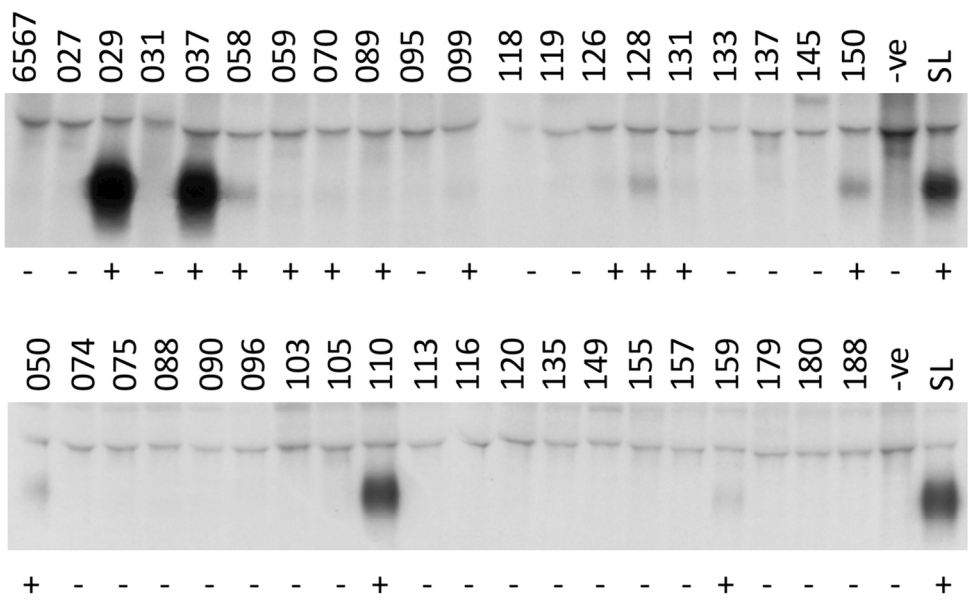


Figure 1
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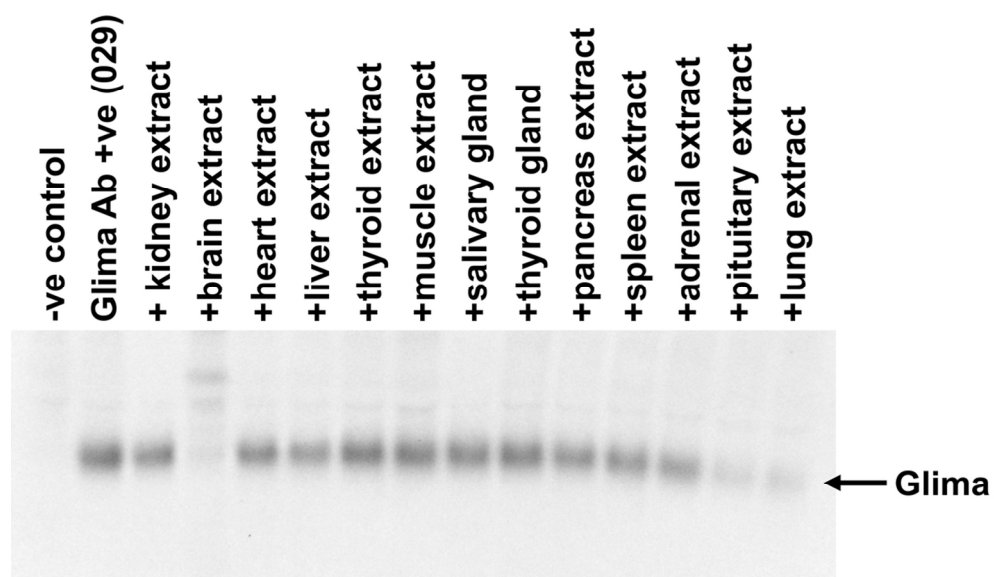


Figure 2
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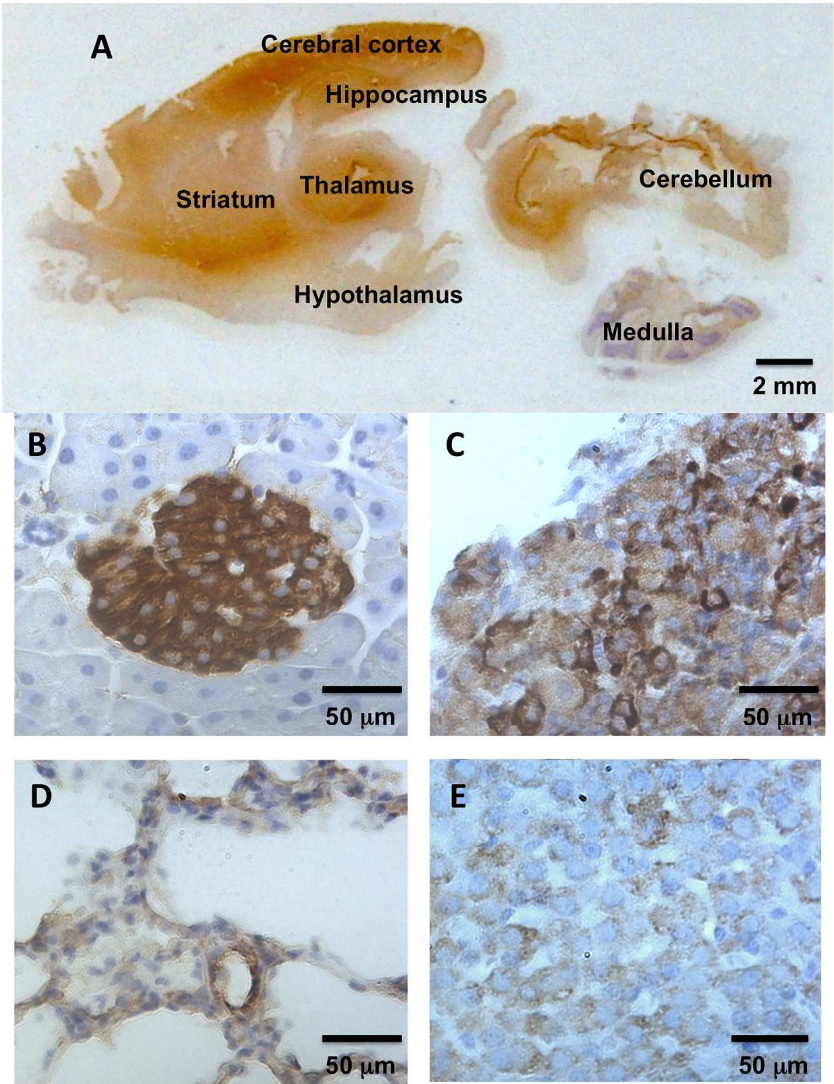


Figure 3
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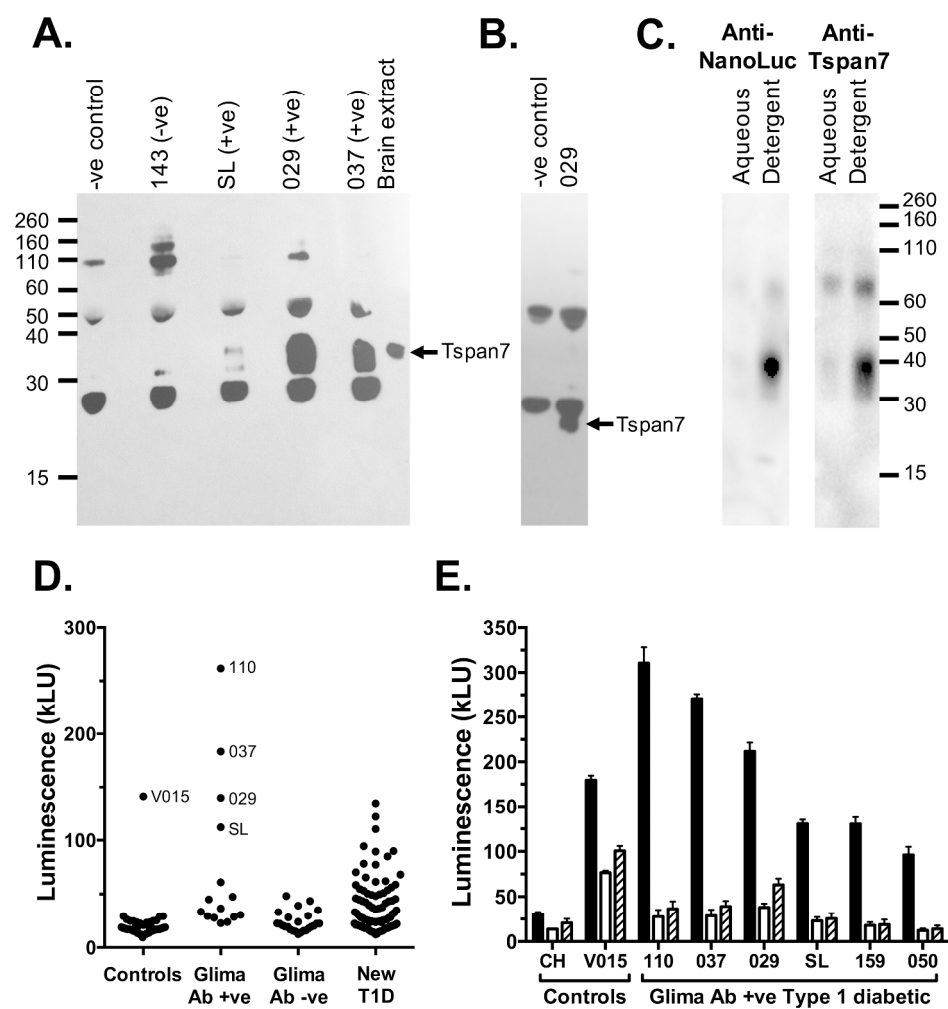


Figure 4
183x187mm (600 x 600 DPI)

Supplementary Table 1: Proteins identified by LC-MS/MS analysis of 38 kD gel purified amphiphilic membrane proteins from mouse brain and lung and from immunoaffinity-purified proteins from brain extracts using bead-immobilised antibodies from Glima antibody positive or negative control sera. Samples in which the protein was identified by LC-MS/MS analysis of the 38 kDa gel slice are indicated in the table by “Yes”. Proteins that were identified in the brain and lung extracts and in the Glima antibody purified sample, but not the negative control are highlighted in bold.

Identified Protein	Accession Number	Molecular Weight	Brain extract	Lung extract	Antibody Purified	Negative control
Actin, cytoplasmic 1 (Actb)	P60710	42 kDa	Yes	Yes	Yes	
Guanine nucleotide-binding protein G(i) subunit alpha-2 (Gnai2)	P08752	40 kDa	Yes	Yes	Yes	
Tetraspanin-7 (Tspan7)	Q62283	28 kDa	Yes	Yes	Yes	
Keratin, type I cytoskeletal 10 (Krt10)	P02535	58 kDa	Yes	Yes	Yes	Yes
Keratin, type II cytoskeletal 1 (Krt1)	P04104	66 kDa	Yes	Yes	Yes	Yes
Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-1 (Gnb1)	P62874	37 kDa	Yes	Yes	Yes	Yes
Keratin, type II cytoskeletal 5 (Krt5)	Q922U2	62 kDa	Yes	Yes	Yes	Yes
Voltage-dependent anion-selective channel protein 2 (Vdac2)	Q60930	32 kDa	Yes	Yes	Yes	Yes
Aquaporin-1 (Aqp1)	Q02013	29 kDa	Yes	Yes		
Sideroflexin-3 (Sfxn3)	Q91V61	35 kDa	Yes	Yes		
Sodium/potassium-transporting ATPase subunit beta-1 (Atp1b1)	P14094	35 kDa	Yes	Yes		
Vesicular integral-membrane protein VIP36 (Lman2)	Q9DBH5	40 kDa	Yes	Yes		
Sideroflexin-1 (Sfxn1)	Q99JR1	36 kDa	Yes	Yes		
Phosphate carrier protein, mitochondrial (Slc25a3)	Q8VEM8	40 kDa	Yes	Yes		
Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-2 (Gnb2)	P62880	37 kDa	Yes	Yes		
Basigin (Bsg)	P18572	42 kDa	Yes	Yes		
ER membrane protein complex subunit 2 (Emc2)	Q9CRD2	35 kDa	Yes	Yes		
Ephrin-B2 (Efnb2)	P52800	37 kDa	Yes	Yes		
Prohibitin-2 (Phb2)	O35129	33 kDa	Yes	Yes		Yes
Keratin, type II cytoskeletal 2 epidermal (Krt2)	Q3TTY5	71 kDa	Yes	Yes		Yes
Sideroflexin-5 (Sfxn5)	Q925N0	37 kDa	Yes		Yes	
BTB/POZ domain-containing protein KCTD12 (Kctd12)	Q6WVG3	36 kDa	Yes		Yes	
Tubulin alpha-1B chain (Tuba1b)	P05213	50 kDa	Yes		Yes	

Guanine nucleotide-binding protein G(o) subunit alpha (Gnao1)	P18872	40 kDa	Yes	Yes	Yes
Glyceraldehyde-3-phosphate dehydrogenase (Gapdh)	P16858	36 kDa	Yes	Yes	Yes
Mitochondrial glutamate carrier 1 (Slc25a22)	Q9D6M3	35 kDa	Yes	Yes	Yes
Synaptophysin (Syp)	Q62277	34 kDa	Yes	Yes	Yes
Sodium/potassium-transporting ATPase subunit alpha-3 (Atp1a3)	Q6PIC6	112 kDa	Yes		
Voltage-dependent calcium channel subunit alpha-2/delta-1 (Cacna2d1)	O08532	125 kDa	Yes		
Myelin proteolipid protein (Plp1)	P60202	30 kDa	Yes		
Sodium/potassium-transporting ATPase subunit alpha-2 (Atp1a2)	Q6PIE5	112 kDa	Yes		
NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 9, mitochondrial (Ndufa9)	Q9DC69	43 kDa	Yes		
Sodium channel subunit beta-2 (Scn2b)	Q56A07	24 kDa	Yes		
Major prion protein (Prnp)	P04925	28 kDa	Yes		
Repulsive guidance molecule A (Rgma)	Q6PCX7	50 kDa	Yes		
Sodium/potassium-transporting ATPase subunit alpha-1 (Atp1a1)	Q8VDN2	113 kDa	Yes		
Coxsackievirus and adenovirus receptor homolog (Cxadr)	P97792	40 kDa	Yes		
Ras-related protein Rab-3A (Rab3a)	P63011	25 kDa	Yes		
Electrogenic sodium bicarbonate cotransporter 1 (Slc4a4)	O88343	121 kDa	Yes		
Sodium channel subunit beta-1 (Scn1b)	P97952	25 kDa	Yes		
Immunoglobulin superfamily member 21 (Igsf21)	Q7TNR6	52 kDa	Yes		
Ras-related protein Rab-10 (Rab10)	P61027	23 kDa	Yes		
Claudin-11 (Cldn11)	Q60771	22 kDa	Yes		
ADP/ATP translocase 1 (Slc25a4)	P48962	33 kDa	Yes		
Electron transfer flavoprotein subunit alpha, mitochondrial (Etfb)	Q99LC5	35 kDa	Yes		
Junctional adhesion molecule C (Jam3)	Q9D8B7	35 kDa	Yes		
Plasma membrane calcium-transporting ATPase 2 (Atp2b2)	Q9R0K7	133 kDa	Yes		
Metaxin-1 (Mtx1)	P47802	36 kDa	Yes		
Translocon-associated protein subunit alpha (Ssr1)	Q9CY50	32 kDa	Yes		
Mitochondrial carrier homolog 1 (Mtch1)	Q791T5	42 kDa	Yes		
1-acyl-sn-glycerol-3-phosphate acyltransferase delta (Agpat4)	Q8K4X7	44 kDa	Yes		

Neuronal membrane glycoprotein M6-a (Gpm6a)	P35802	31 kDa	Yes		
F-box only protein 2 (Fbxo2)	Q80UW2	34 kDa	Yes		
Sodium/potassium-transporting ATPase subunit beta-3 (Atp1b3)	P97370	32 kDa	Yes		
Ephrin-A3 (Efna3)	O08545	26 kDa	Yes		
Cation-dependent mannose-6-phosphate receptor (M6pr)	P24668	31 kDa	Yes		
Protein Dos (Dos)	Q66L44	75 kDa	Yes		
CD99 antigen-like protein 2 (Cd99l2)	Q8BIF0	25 kDa	Yes		
Inactive hydroxysteroid dehydrogenase-like protein 1 (Hsd1l)	Q8BTX9	37 kDa	Yes		
Lipid phosphate phosphatase-related protein type 2 (Lppr2)	Q8VCY8	37 kDa	Yes		
Isocitrate dehydrogenase [NAD] subunit alpha, mitochondrial (Idh3a)	Q9D6R2	40 kDa	Yes		
Myelin-oligodendrocyte glycoprotein (Mog)	Q61885	28 kDa	Yes		
ATP synthase subunit alpha, mitochondrial (Atp5a1)	Q03265	60 kDa	Yes		Yes
Cytochrome b-c1 complex subunit 2, mitochondrial (Uqcrc2)	Q9DB77	48 kDa	Yes		Yes
ATP synthase subunit beta, mitochondrial (Atp5b)	P56480	56 kDa	Yes		Yes
Tetraspanin-8 (Tspan8)	Q8R3G9	26 kDa		Yes	
Corticosteroid 11-beta-dehydrogenase isozyme 1 (Hsd11b1)	P50172	32 kDa		Yes	
Retinoic acid-induced protein 3 (Gprc5a)	Q8BHL4	40 kDa		Yes	
H-2 class II histocompatibility antigen, A-Q alpha chain (Fragment) (H2-Aa)	P04227	24 kDa		Yes	
Syntaxin-1B (Stx1b)	P61264	33 kDa		Yes	Yes
Ig heavy chain V region HPCG13	P01793	14 kDa		Yes	Yes
Haptoglobin (Hp)	Q61646	39 kDa		Yes	Yes
NAD-dependent protein deacetylase sirtuin-2 (Sirt2)	Q8VDQ8	43 kDa		Yes	Yes
Serum albumin (Alb)	P07724	69 kDa		Yes	Yes